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Prolyl Endopeptidase Inhibitory Activity of Two Styrylpyranones from *Phellinus linteus*

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Abstract Two styrylpyranones (1 and 2) were isolated from the CH_2Cl_2-soluble fraction of *Phellinus linteus* and their structures were established by extensive Nuclear magnetic resonance (NMR) spectral data as inoscavin E and C. Both compounds showed significant prolyl endopeptidase inhibitory activity with IC_{50} values of 4.26±0.14 and 4.08±0.04 μM and K_i values of 1.50±0.02 and 1.43±0.03 μM, respectively. They also exhibited antioxidant capacities against the ABTS radical system with EC_{50} values of 6.47±0.05 and 7.64±0.06 μM, respectively.

Keywords antioxidant · inoscavin · *phellinus linteus* · prolyl endopeptidase inhibitor · styrylpyranone

Prolyl endopeptidase (PEP, EC 3.4.21.26, also referred as prolyl oligopeptidase) cleaves proline-containing neuropeptides on the carboxyl side of the prolyl residue in the polypeptides. PEP has been evaluated as a pharmacological target in neurological disease because of its high concentrations in the brain and its ability to cleave peptide hormones and neuropeptides (Gass and Khosla, 2007). Several studies with specific PEP inhibitors in different animal models have found an association of PEP with memory impairment and neurodegenerative disorders (Jarho, 2007).

Dried fruiting bodies of *Phellinus linteus* (a medicinal mushroom known as "sangwhang" in Korea) has been used as traditional East Asian medicine to treat a wide variety of disease including inflammation, allergy, diabetes, gastroenteric disorder, lymphatic disease, and various cancers (Zhu et al., 2008). In our previous effort to search PEP inhibitor from *P. linteus* extract, we reported that davallialactone and inoscavin A showed moderate PEP inhibitory activity with IC_{50} values of 20.0 and 12.2 μM, respectively (Yoon and Paik, 2010). This paper describes two styrylpyranones (1 and 2, Fig. 1) isolated from *P. linteus* extract that exhibit PEP inhibition abilities as well as antioxidant capacities.

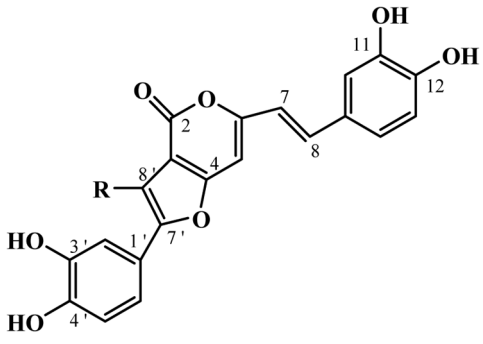

Fig. 1 Chemical structures of compounds 1 and 2.

The ground fruiting bodies of *P. linteus* (600 g) were extracted at room temperature with MeOH. After removal of the solvent under reduced pressure, the combined crude extract was suspended in water and then successfully partitioned to afford EtOAc-soluble and CH_2Cl_2-soluble fractions. The CH_2Cl_2-soluble fraction, which showed PEP inhibitory activity, was subjected to a column of Sephadex LH-20 using 50–70% CH_3CN/H_2O gradient and then rechromatographed on a column of Reversed-Phase C18 columns (ODS) eluting with 50% CH_3CN/H_2O solution. The PEP inhibitory subfraction was subjected to reversed phase HPLC (30–60% CH_3CN/0.1% TFA-H_2O, ODS column) to afford **1** (12.7 mg, R_t 38.2 min) and **2** (7.6 mg, R_t 40.1 min). The structures of compounds **1** and **2** were determined as inoscavin E (Lee et al., 2007; Kojima et al., 2008) and inoscavin C (Lee and Yun, 2006) on the basis of spectroscopic analysis.

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Table 1 NMR spectral data for compounds **1** and **2** (in CD_3OD)[a]

carbon	1			2		
	^{13}C	^{1}H	HMBC (H→C)	^{13}C	^{1}H	HMBC (H→C)
2	161.45			159.94		
3	112.44			109.23		
4	162.86			162.28		
5	96.64	6.83 (s)	3, 4, 6, 7	95.87	6.84 (s)	3, 4, 6, 7
6	158.73			160.00		
7	117.54	6.70 (d 15.8)	5, 6, 8, 9	117.02	6.71 (d 15.8)	5, 6, 8, 9
8	135.69	7.30 (d 15.8)	6, 10, 14	136.69	7.34 (d 15.8)	6, 7, 10, 14
9	129.44			129.08		
10	114.81	7.04 (d 1.9)	8, 11, 12, 14	114.76	7.05 (d 1.6)	8, 11, 12, 14
11	146.96			146.83		
12	148.52			148.61		
13	116.74	6.77 (d 8.2)	9, 11, 12	116.60	6.78 (d 8.2)	9, 11, 12
14	121.81	6.95 (dd 8.2, 1.9)	8, 10, 12	121.90	6.96 (dd 8.2, 1.6)	8, 9, 10, 12
1'	122.49			121.14		
2'	112.80	7.25 (d 2.0)	3', 4', 6', 7'	115.72	7.23 (d 2.0)	1', 3', 4', 7'
3'	147.08			146.58		
4'	148.19			149.19		
5'	116.97	6.83 (d 8.1)	1', 3', 4'	116.45	6.83 (d 8.3)	1, 3', 4', 6'
6'	118.08	7.18 (dd 8.1, 2.0)	2', 4', 7'	121.28	7.17 (dd 8.3, 2.0)	2', 4', 7'
7'	158.48			156.56		
8'	100.47	6.96 (s)	3, 4, 7'	119.23		
9'				198.68		
10'				30.76	2.65 (3H s)	8'

[a]The ^{1}H and ^{13}C data of **1** and **2** were in good agreement with those of reported compounds from *I. xeranticus* (Lee and Yun, 2006; Lee, 2007).

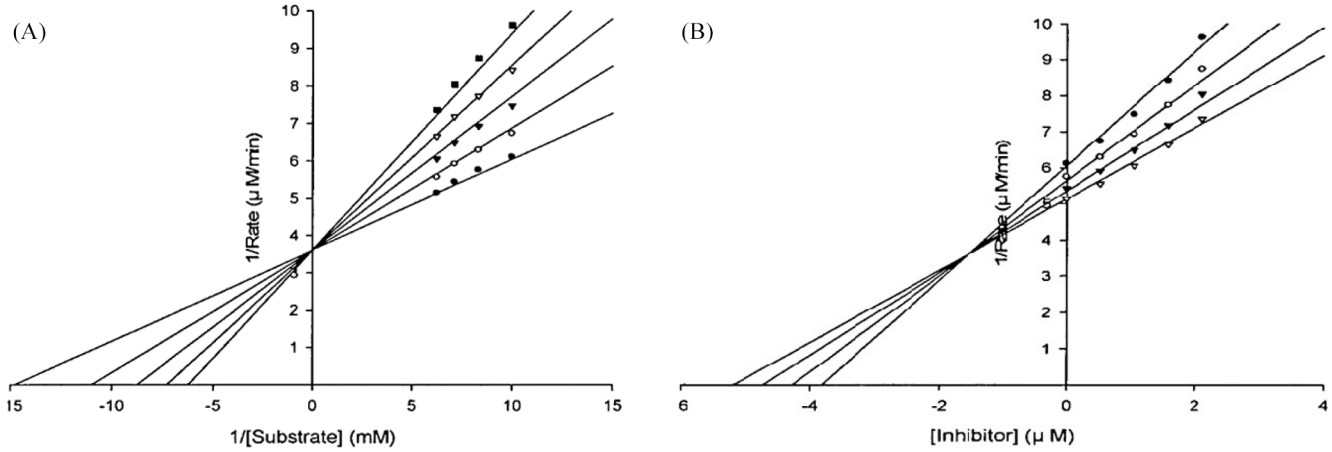

Fig. 2 Lineweaver-Burk plots and Dixon plots of PEP inhibition. (A) Lineweaver-Burk plots of PEP inhibition by **1** [in the absence (●) and presence of 0.53 (○), 1.06 (▼), 1.59 (▽), and 2.12 (■) μM]. (B) Dixon plots of PEP inhibition by **1** [S]=0.10 mM (●), 0.12 mM (○), 0.14 mM (▼), and 0.16 mM (▽).

Compound **1** showed the positive FABMS of $[M+H]^+$ ion at 379.1 and UV absorption maxima at 269 (log ε=3.72), 322 (3.60), and 414 (3.79) nm. The signals of two ABX systems of tri-substituted benzene rings, a *trans*-olefine doublet signals, and two singlet aromatic protons were observed in the ^{1}H NMR spectrum of 1 (Table 1). All signals in the ^{13}C NMR spectrum were sp^2 carbons: 10 of them were methines and the other 11 were quarternary carbons. The ^{1}H, ^{13}C, and HMBC data of 1 (Table 1) were in good agreement with those of reported inoscavin E from fruiting bodies of *Inonotus xeranticus* (Lee et al., 2007). Compound 2 showed the negative FABMS of $[M-H]^+$ ion at 419.1 and UV absorption maxima at 269 (MeOH, log ε=3.79), 416 (3.63) nm. The chemical shifts of ^{1}H and ^{13}C NMR spectra of 2 were in good agreement with those of 1, except for the appearance

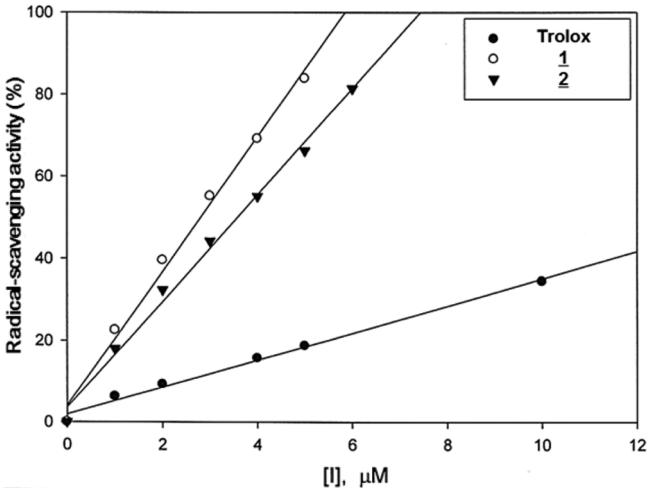

Fig. 3 ABTS radical-scavenging activities of compounds 1, 2 and Trolox.

of an acetyl group (Table 1). The ^{1}H, ^{13}C, and HMBC data of 2 were in accordance with published data of inoscavin C from fruiting bodies of *I. xeranticus* (Lee and Yun, 2006).

The PEP inhibitory activities of 1 and 2 were assayed using benzyloxycarbonyl-glycyl-L-prolyl-*p*-nitroanilide (Z-Gly-Pro-*p*NA) as a substrate, and the amount of released *p*-nitroaniline was determined at 380 nm (Lee et al., 2004). Upon preliminary examination, compounds **1** and **2** at 8 μg/mL inhibited PEP activity almost completely. They were further investigated at various concentrations to evaluate their IC_{50} values. Compounds **1** and **2** showed dose-dependent PEP inhibitory effects with IC_{50} values of 4.26±0.14 and 4.08±0.04 μM, respectively, indicating they were better PEP inhibitors than davallialactone (IC_{50}: 20.0 μM) and inoscavin A (IC_{50}: 12.2 μM) from the same source, *P. linteus* (Yoon and Paik, 2010). The IC_{50} values of these two styrylpyranons (1 and 2) were comparable to those reported inhibitors from other natural plant sources such as ginkgolic acid (0.62 μM, Lee et al., 2004), 8-*C*-(6-*O*-galloyl)glucosylnoreugenin (1.48 μM, Han and Paik, 2012), ursolic acid (17.2 μM, Park et al., 2005), and oleic acid (23.6 μM, Park et al., 2006), suggesting their potential use as bioactive drug against cognitive decline and prevention of memory loss. The Lineweaver-Burk and Dixon plots of the PEP inhibition by compound 1 indicate that it is a competitive inhibitor with K_i value of 1.50±0.02 μM (Fig. 2, Three distinct experiments were carried out). The L-B and Dixon plots of compound 2 also show similar results with K_i value of 1.43±0.03 μM, indicating additional acetyl group of 2 may not function synergistically. The exact mechanism involved in the PEP inhibition by these compounds remains to be elucidated.

Measurement of the radical-scavenging activities of 1 and 2 was carried out using the decolorization of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) radical at 734 nm (Yoon and Paik, 2010). The radical scavenging activities of 1, 2, and trolox (standard reference compound) showed dose-dependent effects with EC_{50} values of 6.47±0.05, 7.64±0.06, and 14.4±0.22 μM, respectively, indicating both compounds have higher free radical-scavenging activity than that of trolox (Fig. 3). The EC_{50} values of 1 and 2 (6.47 and 7.64 μM, respectively) were comparable with the values of reported **1** (22 μM, Lee et al., 2007) and 2 (7.8 μM, Lee and Yun, 2006).

In summary, inoscavin E (1) and C (2) were isolated from the CH_2Cl_2-soluble fraction of *Phellinus linteus*. Both compounds showed significant prolyl endopeptidase inhibitory activity with IC_{50} values of 4.26±0.14 and 4.08±0.04 μM and K_i values of 1.50±0.02 and 1.43±0.03 μM, respectively, suggesting they may have potential to use against cognitive decline and memory loss. They also exhibited antioxidant capacities against the ABTS radical system with EC_{50} values of 6.47±0.05 and 7.64±0.06 μM, respectively.

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